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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	The Institute of Cancer Research Royal Cancer Hospital 17A Onslow Garden London SW7 3AL United Kingdom
Patents ADP number *(if you know it)*	
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom 665295001
4. Title of the invention	BINDING COMPLEXES
5. Name of your agent *(if you have one)*	GILL JENNINGS & EVERY
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Broadgate House 7 Eldon Street London EC2M 7LH
Patents ADP number *(if you know it)*	745002

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Binding Complexes

Introduction

Chaperones are a group of proteins that assist in the folding and refolding of other intracellular proteins. There are many kinds of molecular chaperones HSP100, HSP90, HSP70, Chaperonin (HSP60), DNAJ (HSP40), etc.

One particular family of Chaperones, the Chaperonins, is conserved in all organisms, eukaryotes, archaebacteria and eubacteria alike. The most well studied protein in this family is the eubacteria protein GroEL which has served as a model system for determining the mode of action of the chaperonins.

GroEL exists as a homopolymeric structure in the form of a double ring or toroid structure composed of 7 identical subunits per toroid. The double toroid binds to denatured or partially unfolded proteins and during repeated rounds of ATP hydrolysis achieves the correct folding of the bound protein. The ATPase active site of the individual subunits represents the most highly conserved region of the Chaperonin family of molecules and clearly this function is critical to the activity of Chaperonins from all species.

When examining the primary sequence similarity across the Chaperonin family it is apparent that whilst the ATPase motif is highly conserved (Kim et al 1994, Kubota at el 1995a) outside this region there is only moderate or weak homology between the prokaryotic or endosymbiotically derived type I Chaperonins, GroEL, HSP60 and RBP and the type II Chaperonins of archaebacterium and eukaryotes namely TF55, Thermosomes and CCT (TCP1).

The generally accepted role for GroEL is that it binds to exposed hydrophobic regions of polypeptides that are normally buried within the cores of soluble proteins. By binding to the exposed hydrophobic regions the GroEL prevents aggregation between the unfolded protein monomers themselves or other intracellular molecules. Following substrate binding to GroEL, cycles of ATP hydrolysis drive the progression of the bound substrate towards a folded or near folded state which is then released from the folding complex. GroEL appears to be able to bind to many denatured proteins by means of interaction with hydrophobic pockets or clefts on the surface of the GroEL, indeed GroEL is able to bind to some 50% of denatured cytosolic proteins (Viitanen et al 1992), which suggests a broad specificity for hydrophobic regions in substrate proteins. GroEL mediated folding and release of many substrates is facilitated by the ring co-chaperonin GroES which caps the active cis side of the folding complex (Weissman et al 1996).

By analysis the Type II Chaperonin from eukaryotes, CCT, appears to be an wholly different molecule to GroEL for a number of obvious structural and less obvious mechanistic reasons. CCT is a heteropolymeric complex comprised of eight different subunits in each of two rings which exist as a double toroid structure, the eight subunits being encoded by eight different genes. CCT also appears to bind a far more restricted spectrum of partially folded substrates than GroEL. CCT appears to primarily interact with proteins of the cytoskeleton, namely actin and tubulin, and indeed there are some denatured soluble proteins which CCT will simply not bind (Melki and Cowan 1994). CCT, like GroEL, possesses ATPase activity and the ATPase domain on each CCT subunit is the region showing highest homology with GroEL. There is no GroES like co-chaperonin known for any of the type II chaperonins.

The significantly greater complexity of CCT over and above that of GroEL might suggest that CCT possesses affinity for a wider spectrum of unfolded substrates than GroEL. This does not appear to be the case and therefore an alternate view on the reason for the greater complexity of CCT is that it performs a more complex role within eukaryotic cells than GroEL does in prokaryotic cells. Phylogenetic analysis points to an early divergence of prokaryotic and eukaryotic Chaperonins (Kubota et al 1994) and if CCT evolved at a similar time to the emergence of the cytoskeleton then a specialist actin/tubulin binding function may well have evolved for this Chaperonin family member (Willison and Kubota 1994).

Definitions

"CCT" shall mean the complex comprising CCT subunits α, β, γ, δ, ϵ, ζ, η and θ in the form of a single or double toroid structure described in Kubota et al, Eur J. Biochem (1995) 230 , p3-16.

"CCT micro-complex" shall mean any combination of two or more CCT subunits.

"CCT subunit" shall mean any individual protein encoded by one of the CCT genes Ccta (CCT1), Cctb (CCT2), Cctc (CCT3), Cctd (CCT4), Ccte (CCT5), CctZ1, CctZ2 (CCT6), Ccth (CCT7) or Cctq (CCT8) described in Kubota et al , Eur. J. Biochem. (1995) 230 , p3-16; Kubota et al, Gene (1995) 154 231-236; Kubota et al FEBS LETTERS (1997) 402 53-56.

"conserved CCT microcomplex" shall mean a combination of two or more CCT subunits which are adjacent in the double toroid structure defined in Liou and Willison 1997, (paper in press).

"CCT substrate" shall mean any protein which binds to CCT, CCT micro-complex or CCT subunit during the process of folding into native or semi native state or which binds to the aforesaid at times other than folding into native or semi native state.

"Binding epitope" shall mean the region on the substrate protein or binding partner that interacts with CCT, CCT micro-complex or CCT subunit.

"Substrate peptides" shall mean peptides defining the entire sequence of a substrate protein or binding partner used in the methods to define the binding epitope region.

"Binding epitope peptides (BEPs)" shall mean peptides which define the binding epitope region on the substrate or binding partner.

The Field or the Invention

Substrates for CCT

The vast majority of analysis on Chaperonin substrates has been performed on GroEL, and consequently an appreciation of the breadth of substrates of CCT is more limited. Whilst several known substrates of CCT and CCT analogues have been reported, namely actin, tubulin neurofilament, firefly luciferase, chromaffin membrane components and hepatitis B virus capsid several other legitimate substrates of CCT remain to be identified (Hynes et al 1996). Recent studies have shown that a protein SRB is homologous to CCTδ and may be responsible for binding and enhancing the interaction of TRP-185 with TAR-RNA in HIV infected cells (Wu-Baer et al 1996).

Existence of mini-complexes of CCT

Very little data generated to date has pointed towards the structure, assembly or existence of intermediate sized CCT complexes. There have been two reports which suggest that perhaps CCT subunits act independently of the main 16 subunit double toroid structure.

In Xenopus (Dunn and Mercola 1996) have shown that two subunits (α and_γ) are developmentally regulated and that high levels of expression in the neural crest tissues might represent the site of novel substrates for CCT.

Further evidence of the existence of micro-complexes comes from analysis of CCT in ND7/23 cells undergoing differentiation to a neuronal phenotype. Roobol et al have shown that CCTα enters neuritic processes and co-localises with actin at the leading edge of growth cone structures whereas three other CCT subunits remain predominantly in a perikaryl cytoplasmic region of the cell (Roobol et al 1995).

Isoforms and post translational modification of CCT

CCT is significantly more complex than GroEL in terms of subunit specificity, developmental expression and cellular localisation and recently further evidence of control of activity has come to light with the discovery of a novel post translational modification namely tyrosine adenylylation of CCT. Further evidence of post translational modification has been reported following isoelectric focusing analysis of CCT complexes where evidence of subunit isoforms was evident. If CCT does perform more complex cellular functions than just folding it is reasonable to assume that CCT subunits might be phosphorylated, adenylylated, myrisytolated etc., giving rise to apparent isoforms on 2D gel analysis, a phenomena manifest in proteins which are control points in cellular metabolism.

Specific Field of the Invention

The prior art suggests that CCT performs a different cellular role than GroEL manifest by a more complex subunit composition, isoforms, post-translational modification, differential cellular distribution and the existence of micro-complexes. We present here detailed and novel data that shows evidence of conserved micro-complex structure and a resulting solution to the proposed structure for the toroid. By determining the toroid structure we further propose that distinct substrate binding regions on the complex may also exist which may be utilised in screening for interactions with both known and unknown cellular targets.

Our previous filing (PCT/GB95/00192, WO95/20654) was concerned with cloning, sequence and use of the CCT subunits for the purposes of folding polypeptides. The embodiment presented herein pertains to the function of the complex in binding cellular targets; conserved micro-complexes of CCT; structural determination of the CCT complex; the template directed dis-assembly of CCT; differential expression and cycling of CCT subunits; use of intact CCT, CCT micro-complexes or individual subunits in the identification of substrate binding partner binding epitope peptides (BEPs); use of BEPs in screening for drugs that interfere with cytoskeletal assembly; use of BEPs as therapeutics in their own right; production of antibodies to the BEPs; use of anti-BEP antibodies in screening and use of anti-BEP antibodies as therapeutics in their own right.

Identification of CCT conserved micro-complexes

The enrichment of intact CCT from mouse testis post-nuclear supernatant (PNS) by a 10.2-40% continuous sucrose gradient has shown that this protein complex consistently sediments

in fractions corresponding to 19-23% sucrose (Lewis et al., 1992). The localisation of CCT in the sucrose gradient can be determined through probing, with the monoclonal antibody 91A that recognises mouse CCTα, and a Western blot of the gradient fractions. However, on prolonged exposure of the blot, via enhanced chemiluminescence, the distribution of CCTα extends to fractions corresponding to 10.2-18% sucrose (data not shown). These light sucrose fractions, when resolved by non-denaturing polyacrylamide gel electrophoresis followed by probing with monoclonal antibody 91A, showed several bands in addition to that representing intact CCT (Figure 1A and B). This distribution pattern of CCTα suggests the possible existence of smaller complexes comprising CCT subunits in these sucrose fractions. From here on, these smaller complexes are termed CCT micro-complexes to distinguish them from intact CCT.

Using the alternative technique of Semi-Native Diagonal Electrophoresis (SNaDE; non- denaturing gel electrophoresis in the first dimension followed by SDS-PAGE in the second dimension) and Western blotting, the presence of CCT micro-complexes in sucrose fraction 14 corresponding to sucrose density 1.039 g/cm^3 was examined in detail. Figure 2 shows a single Western blot probed sequentially with eight specific antibodies, recognising different CCT subunits (CCTα, CCTβ, CCTγ, CCTδ, CCTε, CCTζ, CCTη and CCTθ), and with each antibody two signals are observed on the blot consistently. The signal on the left represents intact CCT since a similar and co-incident signal is obtained with all eight specific antibody probings. On the other hand, the signals on the right are generated from CCT subunits present as components of smaller complexes. The non-super-imposability of these signals when probed with different specific antibodies suggest the co-existence of many species of smaller complexes, each comprising a subset of the eight constitutively expressed CCT subunits, CCTα-CCTθ.

The abundance of these various CCT micro-complexes is much less than intact CCT, probably less than 5% in total.

In Figure 2, the complex patterns exhibited on the Western blots, (particularly those in panels F and H) were the result of non-specific cross-reactivity of the polyclonal antibodies against CCTθ and CCTξ used in the analysis. Nevertheless, the signals representing bona fide CCT subunits can be located by comparing their mobilities to the left-most signal representing the corresponding CCT subunit that migrates as a component of intact CCT. In this manner, the signals on the blots representing non-CCT subunits were identified and were excluded during subsequent analysis.

Size distribution of CCT micro-complexes

In order to determine the size distribution of the CCT micro-complexes and ensure that all of them are included in this analysis, sucrose fractions 14-16 (which correspond to sucrose densities of 1.068g/cm^3 - 1.030/cm^3) were pooled and concentrated before being subjected to gel filtration chromatography using a Superose 6 column. Within the 40 resultant fractions obtained, all the CCT subunits were located within the molecular weight range of 5-2700kDa (Figure 3). Similar to the results obtained from the SNaDE analysis, all the CCT subunits were broadly located in two regions. In the first region, centering around fraction 19, a protein complex that has a molecular weight of approximately 920 kDa and is reactive with all the eight specific antibodies, is clearly intact CCT. On the other hand, the distinct cluster of bands in the second region, we attribute to CCT micro-complexes. During each probing with different specific antibodies, the distribution of these bands reflects the size distribution of the subset of CCT micro-complexes containing the respective CCT subunit type. Each CCT subunit type shows a different distribution pattern (Figure 3) demonstrating a distinct size

distribution for the micro-complexes containing each of these subunit types.

From the size distribution of each type of CCT subunit, it is probable that they exist not only as free subunits, but also as components of many larger complexes. To convey this point, we have classified CCT micro-complexes into three categories, (ie. 60 - 100 kDa, 100- 150 kDa, 150-250 kDa) and we infer that they represent monomeric, dimeric and trimeric molecular states (Table 1). The multiplicity of the molecular states for the subset of CCT micro-complexes containing each CCT subunits is obvious. For example, it seems that CCTθ exists only as monomer (i.e. 60 kDa) whereas CCTγ and CCTϵ are found to be present solely as components of larger complexes (i.e. > 120 kDa). The remaining CCT subunits seem to exist as monomers and as components of larger complexes. It is probable that a subset of these complexes may comprise more than one type of CCT subunit.

Again, due to the non-specific cross reactivity for some of the polyclonal antibodies used, it is essential to identify the signals representing CCT subunits in order to ensure the accuracy of subsequent analysis. This was done by taking an identical gel lane containing all these bands and resolving them by SDS polyacrylamide gel electrophoresis in the perpendicular direction (data not shown). All bands that contain CCT subunits produce signals at their corresponding molecular weights. For example, any micro-complexes containing CCTα will yield a signal in the 57 kDa region after they are resolved by SDS-PAGE. In this manner, all bands observed on the non-denaturing gel lanes that were not comprised of CCT subunits were determined and were not considered during further analysis. However, all the signals that seem to represent bona fide CCT micro-complexes and which were superimposable provided the subunit association patterns.

For clarity, all the overlapping signals are indicated by inset arrows and each association between different types of subunit is indicated. In Table 2, all the observed subunit-subunit association patterns are tabulated.

Determination of the Subunit Orientation for the torodial ring in CCT

Analysis, based on the protein sequence of the CCT subunits and the structure of GroEL (Kim et al., 1994), predicts that each type of CCT subunit will only associate to two other different types of subunit within each ring of CCT. As predicted, each type of CCT subunit associates only to one or two other different types of CCT subunit (Table 2) with the exception of CCTθ which remains mainly as a free subunit. This feature of association specificity for each CCT subunit type suggests a unique orientation for the subunits forming the ring.

From Table 2, there are sufficient sets of association patterns to enable the construction of a probable subunit orientation within each CCT ring (Figure 5). However, in mouse testis, the absence of an observable association pattern between CCTγ and CCTβ gives rise to an additional possible arrangement for the subunits in the ring.

Nevertheless, the association of these two subunit types observed in human 293 cells (Figure 4, panel J and K) allows discrimination between the two alternative arrangements from the mouse testis data. Analysis of sucrose gradients from cellular extracts by western blotting has proven the existence of the 920 KDa CCT complex comprising all 8 subunits in the double toroid structure. More detailed analysis across the sucrose gradient by western blotting with enhanced chemiluminescence has revealed the existence of many "micro-complexes" with lower molecular weights comprising monomeric, dimeric and trimeric combinations of the

individual subunits. From extensive analysis of various tissues with antibodies to the individual subunits it is apparent that there are preferred or conserved combinations in these micro-complexes. Analysis of CCT subunit mutants in Yeast (Vinh and Drubin 1994) suggests that CCT subunit δ is implicated in actin binding, whilst CCT subunits α,β (Miklos et al 1994, Chen and Huffaker 1994) are predominantly involved in tubulin binding. This is consistent with a unique structure whereby different substrate binding regions are spatially separated.

The existence of micro-complexes also suggests and supports the idea that individual or combinations of the CCT subunits might perform specific binding functions in their own right and that the CCT complex is a holding structure to enhance productive binding with substrate due to the higher regional concentration and geometry of subunits within the complex. If this is the case it would support earlier hypotheses that CCT and its individual subunits might perform significant control functions within the cell. Given the importance of CCT's substrates actin and tubulin and CCT binding partners cyclins D1, D2 and E in the function of the cell, it is equally reasonable to expect that CCT and its subunits may play pivotal control or checkpoint functions by binding to interactive regions of the substrates and binding partners.

As a direct result of discovering the existence of micro-complexes we have been able to fit a solution to the subunit organisation of the intact CCT complex. With 8 different subunits and 2 toroids there are potentially 40,320 possible combinations and 5040 combinations for a single toroid that might exist, however, by exhaustive analysis of the conserved micro-complexes there is only 1 solution to the CCT structure which satisfies the adjacent neighbour data disclosed. Figure 5 portrays the subunit structure of one toroid of the intact CCT complex.

With the unique knowledge of this structure we expect the possibility of using the complex in soluble or immobilised form to probe for target BEPs and the possibility of using the intact CCT complex as a molecular vice, to hold recombinant or synthesised proteins for presentation to molecular probes that bind to folding intermediates held between known BEPs bound by interactions with, for instance, diametrically opposite binding subunits on the CCT toroid or even adjacent positions.

Differential subunit cycling into the CCT complex

As discussed above, biochemical analysis of the CCT complex indicates that it has a unique subunit structure and composition. However, pulse-labelling with ^{35}S-methionine of CCT complex in vivo appears inconsistent with this model because individual subunits do not label at similar rates and the following experiment provides quantitative data for supporting this hypothesis. Germ cell preparations were labelled for 1 hour with ^{35}S-methionine and the 20S sucrose CCT peak (Lewis et al., 1992) was analyzed by 2-D PAGE (Fig. 6a, b). We have established a reference profile of the polypeptide composition of the 20S sucrose CCT peak using a combination of protein sequencing (Kubota et al., 1994), immunoblot analysis with antibodies to CCT subunits and substrates (Hynes et al., 1995) and peptide mass fingerprinting (Hynes et al., 1996). The relative quantities of each CCT subunit observed by silver staining (Fig. 6a) and autoradiography are not equivalent (Fig. 6b); CCTα and CCTϵ (S2 & S3) have higher specific activities than the other subunits (normalized data in Fig 6 legend) and CCTξ (S7) has very low specific activity. Fig 6c shows CCT labelled for 1 hour as above, but followed by a chase in the absence of ^{35}S-methionine for a further 4 hours. As expected, the substrates on CCT which are strongly labelled after a 1 hour chase period (Fig. 6b) have decreased activities after the end of the chase period (Fig 6c); however, CCT subunits become more

stoichiometrically labelled after the 4 hour chase. The combined data from these in vivo labelling experiments demonstrate that CCTα and CCTϵ, when incorporated into core CCT, are more heavily labelled compared to other subunits. This suggests that CCT subunits are in equilibrium between the main CCT complex and other pools of subunits and that, during the course of a 1 hour labelling period, the subunits of intact CCT must be turning over or cycling.

Further evidence of a complex and dynamic structure for CCT comes from this analysis of the rates and stoichiometry of CCT subunit synthesis. Were the CCT complex a unitary reactive folding centre akin to GroEL one might expect equivalent rates of synthesis of the individual subunits.

Analysis has revealed that in certain tissues there is a 5 fold range of mRNA levels between the various subunits and as a consequence of this there is a five fold range of labelling rate of the intact complex with ^{35}S-labelled subunits. This data support the hypothesis of a highly dynamic fluxing CCT complex in which subunits and micro-complexes are constantly moving into and out of the "holding" complex in response to substrate binding, ring disassembly, ATP hydrolysis and reassembly.

Substrate mediated disassembly of the CCT complex

During time course translation experiments primed with the β-actin gene as template we observed that newly synthesised actin formed a binary complex with CCT as previously reported (Gao et al 1992). We observed an additional band migrating between 443 and 886 kDa which becomes labelled at 15 minutes suggesting the existence of a newly discovered intermediate between actin and CCT subunits or micro-complexes. Verification of the presence of CCT subunits in the intermediate by immunoblotting has proved difficult both due to its transient nature and the inherently low quantity generated during the expression of β-actin.

Furthermore, the instability of the β-actin intermediate in high salt (300-400 mM NaCl) rendered purification by anion-exchange chromatography unfeasible with typical losses greater than 70% (data not shown). Nevertheless, some success was achieved in concentrating this intermediate based on its sedimentation in a continuous 10-40% sucrose gradient. After gradient fractionation, each fraction was resolved by 6% native polyacrylamide gel electrophoresis and Western blotted. Consistently, the intermediate resided in sucrose fractions 13 and 14 (which correspond to sucrose densities 1.059-1.053 g/cm^3) whereas CCT was located in fraction 11 (1.0806 g/cm^3) (Figure 7A) as indicated by autoradiography. When the same blot was probed with a polyclonal antibody UM1, which recognises all the mouse CCT subunits (Hynes et al., 1995), the intermediate was found to cross-react with this antibody (Figure 7B). This result suggests that a subset of the CCT subunits may be part of the intermediate. In addition, the strong smear in fraction 14 suggests the co-existence of smaller complexes containing CCT subunits (CCT micro-complexes). Both observations, in concert, support a model whereby the folding human β-actin is mediated by CCT ring disassembly, producing the actin intermediate and other CCT micro-complexes.

Association of individual subunits on microcomplexes after complex disassembly

The results support a model whereby the folding of β-actin involves ring disassembly. In addition, we also observed that individual CCT subunits and/or CCT micro-complexes are generated from the disassembly process, which is consistent with the CCT micro-complexes detected in mouse testis lysate (Liou and Willison, 1997). Furthermore, CCTδ and CCTη respectively, were present as two distinct species after the ring disassembly process indicated by differences in their migration distance in native PAGE of the SNaDE analysis. The one on the right, smaller in size, probably

represents their existence in micro-complexes or as monomers, whereas the other on the left, higher in molecular weight (indicated by arrow heads in panel F of Figure 8 and panels E and F of Figure 9), may indicate their existence in the actin intermediate. In other words, these two CCT subunits seem persistently attached to the folding β-actin even after the CCT ring disassembly processes has occurred which is consistent with the probing of the β-actin intermediate by the polyclonal antibody UM1, which also indicated the presence of CCT subunits (Figure 7).

Semi Conservative CCT Ring Assembly

We have previously described an anti-mouse TCP1/CCTα monoclonal antibody, 23C (Willison et al., 1989) which fails to bind human TCP-1 (Lewis et al., 1992) and TCP-1 of other primates and Xenopus laevis (Hynes et al., 1996). All mammalian TCP-1 genes sequenced so far contain 556 residues and are 96% identical with mainly the extreme N and C-termini showing a significant degree of sequence divergence between species. The 23C monoclonal antibody binding site on mouse TCP-1/CCTα has been mapped to an epitope 'LDD' which is situated as the last three residues of the mouse CCTα. The reason for the absence of binding of 23C to a single residue change in the 23C epitope region in human TCP-1, D555N (Harrison-Lavoie et al., 1993; Hynes et al., 1996), making the last three residues as 'LND'. In the case of rabbit CCTα, we ascertained that the last three residues were 'LLD' by PCR amplification of a rabbit brain cDNA library (Figure 10A).

Subsequently, a D555N mutation was introduced into mouse CCTα to remove the 23C antibody binding site giving rise to a mutant encoding mouse CCTα with 'LND' as the C-terminal end. Consequently in rabbit reticulocyte lysate, rabbit CCT should bind to 2 molecules of 23C and if the rabbit CCTα is exchanged for the corresponding mouse CCT subunit

by its expression in vitro, the labelled CCT should still bind 2 molecules of 23C. If however, the LND mutant CCTα is expressed and incorporated into rabbit CCT, there are two possible outcomes with respect of 23C binding depending on the mechanism in play. If there is complete disassembly and reassembly of CCT, all the newly assembled labelled CCT should contain 2 'LND' CCTα subunits. However, if the assembled labelled CCT contains only a single 23C binding site, then there must be single ring disassembly and reassembly.

By introducing antibody 23C into a rabbit reticulocyte lysate mix containing expressed mouse CCTα, the presence of a shift in migration distance of CCT is observed (Figure 10B, lanes 1 and 2). However, when the D555N mutant mouse CCTα is expressed, the retardation of CCT migration was only half of that observed with wildtype mouse CCTα (Figure 10B, lane 4). This lessening of shift to half the migration distance is attributable to a single antibody molecule only being coupled onto CCT. This result implies that the incorporation of CCTα is one ring at a time at every reassembly cycle. A pictorial representation of the endogenous CCT coupled with two antibody molecules of 23C and mutant CCT coupled only to one antibody molecule of 23C is shown in Figure 10C. In summary, this result indicates that the incorporation of subunits into CCT occurs singly. By extrapolation, the disassembly process is also expected to occur in single ring fashion, meaning that only the ring that was occupied by the substrate is disassembled during the folding cycle.

Use of CCT complexes to identify immobilised Binding Epitope Peptides (BEPs) on CCT substrates

Prior art has shown that certain domains within substrate proteins are responsible for the interaction with CCT. Dobrzynski et al have defined an internal M domain in β-tubulin spanning some 120 amino acids which interacts

strongly with TRiC and a further 140 "N" terminal amino acids which interact less strongly with TRiC. Previously one might have expected that large tracts of proteins generally representing the hydrophobic core of soluble proteins are responsible for the interaction with chaperonins, unexpectedly in this embodiment we find that small peptides spanning 15 residues are capable of high affinity binding to CCT complexes. Further we have discovered that there are numerous binding sites along substrate molecules, identifying what we term binding epitope peptides, (BEPs) and that there are specific clusters of BEPs along the substrate molecule. By panning the entire substrate molecule, in this case actin, in 15 residue portions, we have identified not only BEPs but also hot spots or clusters where there are clearly enlarged epitopes which encompass 30-40 residues of high affinity binding.

Peptides of 15 amino acids in length were synthesised on polyethylene pins mounted on blocks according to the method of Maeji et al (Maeji N.J. et al 1994) and commercially available under the trade name of Pepsets ™ from Chiron Mimotopes. The sequence of the peptides were such that they defined the full length of the actin sequence with a 5 amino acid overlap between the sequential peptides, and selected peptides representing key known structures in actin (Table 3 annotated). The peptides are synthesised on polyethylene pins which are presented to be compatible with standard 96 well microtitre plates. The pins become the solid phase on which interactions with CCT complexes can be probed. The method is described below.

1. Take Pepset pins from storage at -20(°C) and equilibrate at room temperature.
2. Wash pins with PBS for 10 minutes at room temperature on a shaking table.

3. Incubate pins with blocking buffer (2% w/v BSA 0.1% Tween 20 in PBS) for 60 minutes at room temperature on a shaker.

4. Wash pins with PBS for 10 minutes at room temperature on a shaker (times 1)

5. Take 20S mouse testis sucrose gradient fractions enriched in CCT and make up to a volume of 100 ml with binding buffer (0.5 mM $MgCl_2$ in breaking buffer pH7.2)

6. Incubate pins with CCT solution overnight at $4^{\circ}C$ on a shaker

7. Continue incubating the pins at room temperature with the CCT solution for one hour at room temperature on a shaker.

8. Wash pins with PBS for 10 minutes at room temperature on a shaker (times 3).

9. Incubate pins with a solution of 91a, monoclonal antibody to CCTα, (5ul of Affinity Bioreagents stock 91a in 100 ml of PBS) for 2 hours at room temperature on a shaker.

10. Wash pins with PBS for 10 minutes at room temperature on a shaker (times 3).

11. Incubate pins with anti-rat CAP (Pierce product No. 31350) (5ul in 100 ml PBS) for 2 hours at room temperature on a shaker.

12. Wash pins with PBS for 10 minutes at room temperature on a shaker (times 3).

13. Dispense 200ul pNPP liquid substrate (Sigma product No. N7653) into each well of a 96 well microtitre plate (Immulon product No. M129A11-50).

14 . Invert pins into microtitre plate and incubate with pNPP reagent for 30 minutes at room temperature in the dark.

15. Remove pins from the microtitre plate and read the microtitre plate at 410nm on a Dynatech ELISA plate reader.

16. Results are expressed as a 410nm absorbance reading for each well corresponding to each unique substrate peptide from the actin molecule.

Figure 11 Shows the 410nm absorbance reading obtained versus the substrate peptide number for the actin molecule; unexpectedly there are unique hot spots of binding along the actin molecule which define the binding epitope regions for CCT. The sequence of the BEPs for actin are displayed in Table 3 and show that the BEPs for actin are not exclusively comprised of hydrophobic peptides but a mixture of hydrophilic and hydrophobic peptides with differing charges. This suggests that these BEPs are unique binding sites for CCT which are highly specific and probably bind to differing subunits of CCT or differing regions on individual CCT subunits. Further the hot spot of BEPs situated at substrate peptides 6-8 is the major surface binding region of actin for DNAse 1 clearly showing that CCT; i) does not exclusively bind to hydrophobic inner cores like GroEL; ii) binds to surface residues accessible to aqueous solution; and iii) binds to regions of proteins implicated in non-CCT protein-protein interactions of significance in intracellular processes. Further, there appears to be two or three types of interaction site or epitope as gauged by the strength of CCT binding to the immobilised peptides; primary sites, reference peptide numbers 1, 2, 3 and 4(Figure 12); secondary sites, reference peptides 6, 7 and 8 (Figure 12); and tertiary sites, reference peptides 9, 10, 11, 12, 13, 14 and 15 (Figure 12). These may truly represent secondary or tertiary interaction sites or may be equally strong binding epitopes that are conformationally restricted or cleaved during the immobilisation process. Such secondary or tertiary interaction sites should be probed in alternative procedures to determine the strength of binding to CCT.

If CCT's role within the cell is to prevent unscheduled interactions between proteins that are folding or merely residing as intracellular pools, then it is reasonable to assume that key interactive sites of proteins such as actin, tubulin and even the cyclins will be recognised by

CCT to prevent such non productive interactions. The experiment described above has an internal positive control in the major DNAse 1 binding site which is one of the major binding sites for actin. The methodology described has indeed identified a major protein-protein interaction site on actin, but unexpectedly has also identified a new protein interaction site in peptide 61 (Figure 11) the role of which has yet to be elucidated. We anticipate therefore that the methods described will enable hitherto unrecognised sites of modulation and protein-protein interaction to be identified on CCT substrates and binding partners.

Immobilised BEPs identified by CCT are also recognised in free solution

The molar concentration of BEP immobilised on the pin used in the screening assay is extremely high and potentially may have no relevance to the interaction between the actin molecule and CCT in the cytosol. Therefore we incubated CCT with biotinylated actin peptide number 1, 2 and 3 (Figure 12) in buffered solution and then subjected the complete mixture to non denaturing gel electrophoresis which separates the non-binding peptides from CCT-bound peptides. The gel was western blotted and probed with streptavidin-HRP conjugate to determine the position of the biotinylated peptides (Figure 13). The biotinylated peptides, numbers 1, 2 and 3 (Figure 12) were shown to co-migrate with the CCT complex, whereas control peptides did not verify that significant and specific binding between CCT and BEPs identified in the immobilised assay occurs in free solution.

A further analysis was performed with a derivative of peptide 8 (Figure 11) wherein alanine substitutions where made within the peptide to try and further identify the critical residues responsible for CCT binding (Figure 13). We have found that substitution of the sequence GRPRH by

alanine residues within peptide 8 significantly depletes binding to CCT in this free solution assay. This further confirms the ability of the methodologies embodied herein to identify protein-protein interaction sites, to find the minimal number of residues responsible for binding within a BEP and to perform mutation analysis on the BEPs to modify the efficacy of BEP binding.

The positive BEPs identified in the immobilised assay also bind with significant affinity in free solution, supporting the premise that these peptides do indeed represent "binding epitopes" of relevance in the cytoplasmic setting.

Inhibition of substrate binding to CCT by BEPs

For BEPs identified for various CCT substrates to be of any use they must efficaciously compete with native or folding polypeptide for occupancy of the CCT complex, CCT micro-complex or CCT subunit. A simple competition assay has been developed whereby an actin BEP is incubated with CCT complex and actin mRNA primed rabbit reticulolysate to determine if the BEP can inhibit productive binding between newly synthesised actin and CCT.

Micro-injection of BEPs into living cells has profound physiological effects

Previous studies have shown that injection of antibodies to CCT into living cells has a profound effect on microtubule mediated events (Brown et al 1996).), in this case centromere function was profoundly disrupted as a result of the inability of CCT to assist in the folding of tubulin. This work suggests that CCT is critically implicated in centromere function whereas another chaperone hsp73 is less critical in this setting, since microinjection of anti-hsp73 antibodies did not illicit the same response as anti-CCT antibodies.

BEPs isolated from actin when injected into cells will have a significant effect on actin mediated events such as membrane ruffling and other cellular motion events mediated by the actin cytoskeleton. The BEPs will compete with newly synthesised actin for binding to CCT which will result in incomplete folding of actin thereby disabling the cell from assembling native actin that has been newly synthesised.

Therapeutics based on BEPs

The unexpected discovery that substrate proteins for CCT possess restricted multiple hot spots of binding which correspond to critical regions of protein-protein interaction has significant implications on the design of new therapeutic molecules for many diseases.

The CCT BEPs identified on the actin molecule represent highly specific "epitopes" of protein-protein interaction which are distinctly different to the tracts of hydrophobic amino acids which characterise the non-specific binding regions for GroEL and its substrates. This observation supports other data which shows that GroEL will bind to most denatured proteins whereas (Viitanen et al 1992) many denatured substrates will not bind to CCT (Melki et al). This suggests that the interaction between CCT and its substrates is more specific and may be responsible for a control function in cellular physiology.

Some 5-10 BEPs on actin have been identified using the methodologies disclosed above and the amino acid composition of these BEPs shows that, unlike folding cores for model proteins such as Barnase which are predominantly hydrophobic and indeed non-specific, the BEPs for actin are equally mixed hydrophobic and hydrophilic peptides. We anticipate that in the design of therapeutics the BEPs identified by the disclosed methods may need significant optimisation to illicite stronger binding. The embodiment

herein also discloses the concept of using antibodies against the initial BEPs to screen peptide libraries, whereby the anti-BEP antibody replaces CCT , CCT microcomplexes or individual CCT subunits in the screening assays to seek stronger more potent binding partners for CCT.

The unexpected finding of relatively small binding epitopes on CCT substrates suggests that CCT might be used to identify regions on substrate proteins which are involved in protein- protein interactions other than with CCT. Whereas those skilled in the art use panels of monoclonal antibodies or degenerate peptide libraries to identify protein-protein binding sites on known interacting proteins, the methodology described herein offers the potential to identify protein-protein interaction sites between the CCT substrate molecule and other unknown proteins by virtue of identifying the BEPs for CCT binding. Protein sites that bind strongly to CCT are obvious candidates for binding to other proteins and furthermore may not be obvious binding sites if they are buried in the native state. Since CCT recognises folding intermediates and indeed CCT micro-complexes remain bound to folding intermediates after CCT disassembly, it is reasonable to assume that BEPs for CCT may represent conformational epitopes not normally available for solution phase binding to the native protein, being only available after conformational changes to the protein.

Not only do we anticipate identifying new protein-peptide therapeutics using the BEP panning methodology described above but also therapeutics which are specifically designed to target actively synthesising cells. BEPs which are identified as binding to CCT will compete with newly synthesised polypeptides for CCT binding rather than existing pools of CCT substrates. This is a significant advantage over drugs which interfere with tubulin by

stabilising or disrupting the microtubules. Inevitably for anti-tumour drugs there is significant toxicity associated with their use since all cells will be susceptible to drugs such as taxol and vincristine that stabilise microtubules and it is only by virtue of the fact that actively dividing cells require constant turnover of microtubules to achieve replication that tumours are marginally more susceptible than normal tissue. We therefore anticipate that BEP, BEP mimics and small molecules that compete with BEP binding to CCT will represent novel therapeutic candidates which will target cells active in protein synthesis, since inhibitors of BEP binding to CCT are specific for substrate proteins that are folding after synthesis, rather than existing pools of folded CCT substrates.

New substrates for CCT binding

It is well reported in the literature that the known substrates for CCT are actin and α, β and γ tubulins. Accordingly BEPs identified for these known CCT substrates are obvious candidates for the identification of new therapeutic candidates. We have already disclosed in the prior art that there are many other unknown substrates that specifically bind to CCT at the time of their synthesis. We now disclose that Cyclin E, D1 and D2 are specific binding partners for CCT with binding kinetics which are significantly different to kinetics for folding substrates such as actin or tubulins (Figure 14). This is significant in that specific binding partners have been identified that bind in a manner not concomitant with folding but which nevertheless bind specifically. This opens up the possibility that protein binding to CCT, CCT micro-complexes or CCT subunits could act as a control mechanism preventing binding to other non-CCT subunits therefore placing CCT in a pivotal control function for cellular processes. Our data on BEPs from actin showing that a major binding epitope is the surface positioned major

binding region for DNAse 1 confirms that BEPs for CCT are likely to be significant interaction points for protein-protein interaction with non-CCT proteins. We therefore anticipate the identification of BEPs for these proteins and subsequently peptide, peptide mimetics, antibodies, antibody fragments and small molecule inhibitors of CCT binding that cause therapeutic effect through interaction with Cyclin E, Cyclin D1 and D2 directly or by competing with their binding with CCT and other cellular proteins.

CLAIMS:

1. A peptide fragment of a protein substrate of the CCT complex, capable of occupying the protein substrate binding site on the CCT complex, a CCT micro-complex or a CCT subunit.

2. A peptide capable of occupying the substrate protein binding site on the CCT complex, a CCT micro-complex or a CCT subunit, without the peptide undergoing folding on the CCT complex, micro-complex or subunit.

3. A peptide capable of binding to the CCT complex or CCT micro-complex, to cause disassembly, without the peptide undergoing folding on the CCT complex or micro-complex.

4. A peptide according to any of the previous claims, wherein the peptide is up to 40 amino acids in length.

5. A peptide according to any preceding claim, wherein the peptide is from 5 to 15 amino acids in length.

6. A peptide according to any preceding claim, wherein the peptide is a fragment of actin.

7. A peptide according to any of the previous claims, wherein the peptide has at least part of an amino acid sequence shown in any of Reference Peptide Nos. 1 to 15.

8. A peptide according to any of the previous claims, wherein the peptide has the amino acid sequence GRPRH.

9. Use of a peptide capable of occupying the protein substrate binding site on the CCT complex, a CCT micro-complex or a CCT subunit, to inhibit the folding of a protein substrate on the CCT complex, micro-complex or subunit.

10. Use according to claim 9, wherein the peptide is as defined in any of claims 1 to 8.

11. Use of the CCT complex, a CCT micro-complex or a CCT subunit to identify regions on proteins that undergo protein-protein interactions other than with the CCT complex, micro-complex or subunit.

12. A method for identifying an inhibitor of protein folding in eukaryotes, wherein the CCT complex, a CCT micro-complex or a CCT subunit is probed with a peptide.

13. A method according to claim 12, wherein the peptide is up to 40 amino acids in length.

14. A method according to claim 12 or claim 13, wherein the CCT complex, micro-complex or subunit is immobilised on a solid support.

15. A method according to claim 12 or claim 13, wherein the peptide is immobilised on a solid support.

16. A method according to any of claims 12 to 15, wherein the CCT complex, micro-complex or subunit is labelled for detection.

17. A method according to any of claims 12 to 15, wherein the peptide is labelled for detection.

18. A method according to claim 16 or claim 17, wherein the label is a reporter molecule.

19. A method according to claim 16 or claim 17, wherein the label is an antibody or antibody fragment.

20. A method according to any of claims 12 to 19 wherein the inhibitor is identified by detection of the disassembly of the CCT complex or micro-complex, or by the detection of released subunits.

21. Use of a recombinant CCT complex, a CCT micro-complex or a CCT subunit in any of the methods defined in claims 12 to 20.

22. An antibody to a peptide defined in any of claims 1 to 8.

23. Use of an antibody defined in claim 22, to identify a peptide capable of binding to the CCT complex, a CCT micro-complex or a CCT subunit.

24. Use of an antibody or antibody fragment that inhibits binding of a protein substrate to the CCT complex, a CCT micro-complex or a CCT subunit for therapeutic use.

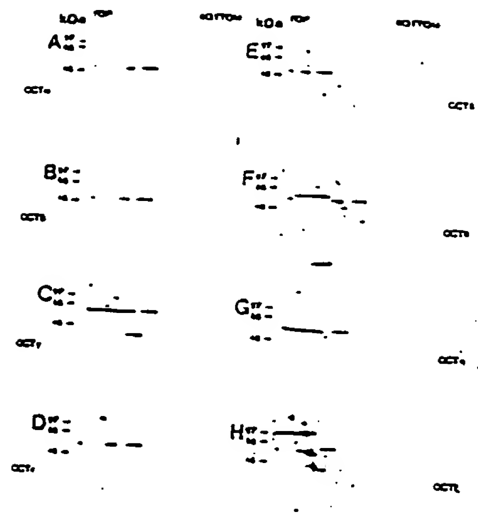

Figure 2 The presence of CCT micro-complexes in sucrose gradient fraction 14 (corresponds to sucrose density 1.039 g/cm^3) from analysis by Semi Native Diagonal Electrophoresis (SNaDE) and Western blotting.

Semi Native Diagonal Electrophoresis (SNaDE) analysis of the mouse testis sucrose fraction 14 shows the presence of intact CCT (left most spot on the blot) and CCT micro-complexes (indicated by inset arrows). The 'Top' and 'Bottom' in the figure represent the orientation of the 6% non-denaturing gel slice that was further resolved in an 8% SDS PAGE gel. A Western blot of the SDS PAGE gel was probed sequentially with specific antibodies indicating the distribution patterns of A) CCTα; B) CCTβ; C) CCTγ; D) CCTε. Another identical Western blot was probed sequentially with the other four specific antibodies indicating the distribution patterns of E) CCTδ; F) CCTθ; G) CCTη and H) CCTζ.

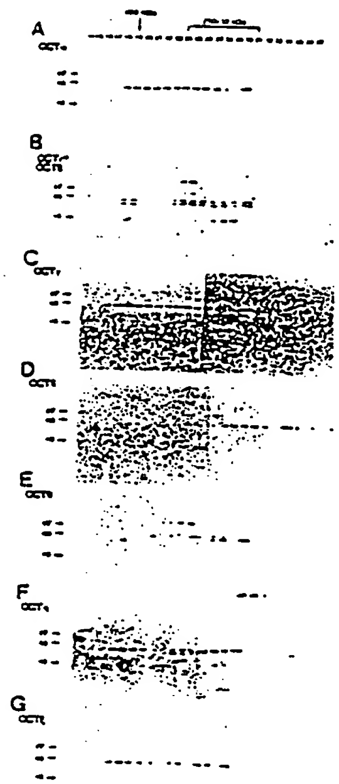

Figure 3 Molecular size distribution of each CCT subunit type when present as components of CCT micro-complexes.

CCT micro-complexes present in sucrose fractions 14-16 (corresponding to sucrose densities 1.039 g/cm^3-1.030 g/cm^3), were separated by gel filtration chromatography and the chromatographic fractions 14-37 (corresponding to molecular weight range of 2700-5 kDa) were resolved in an 8% SDS polyacrylamide gel followed by Western blotting. The blot was probed sequentially with specific antibodies recognising different CCT subunit to indicate their distribution patterns. Panels A-G showed the molecular size distributions of CCT micro-complexes containing CCTα; CCTβ and CCTε; CCTγ; CCTδ; CCTθ; CCTη; and CCTζ respectively. The band observed in fraction 19 which cross-reacted with all the specific antibodies used to identify CCT subunits and which has an estimated molecular weight of 920 kDa is intact CCT.

Table 1 Multiplicity of Molecular States of CCT Micro-Complexes containing each CCT subunit type

Sizes	Types of CCT subunit in Micro-Complexes							
kDa	CCTα	CCTβ	CCTγ	CCTε	CCTδ	CCTθ	CCTη	CCTζ
60 - 100	+	+	-	-	+	+	+	+
100 - 150	+	+	+	+	+	-	+	+
150 - 250	+	+	+	+	-	-	+	+

+ Subunit present in complexes of respective size class
- Subunit absent in complexes of respective size class

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Table 2 Subunit-Subunit Association Patterns observed in CCT Micro-Complexes

	CCTα	CCTβ	CCTγ	CCTε	CCTδ	CCTθ	CCTη	CCTζ
CCTα	n.d.							
CCTβ	-	n.d.						
CCTγ	-	+[a]	n.d.					
CCTε	+	-	-	n.d.				
CCTδ	-	-	-	-	n.d.			
CCTη	+	-	-	-	+	n.d.		
CCTθ	-	-	-	-	-	-	n.d.	
CCTζ	-	+	-	+	-	-	-	n.d.

[a] This association is not very clear in mouse testis but substantiated in Human 293 cells

n.d. : Not Determined

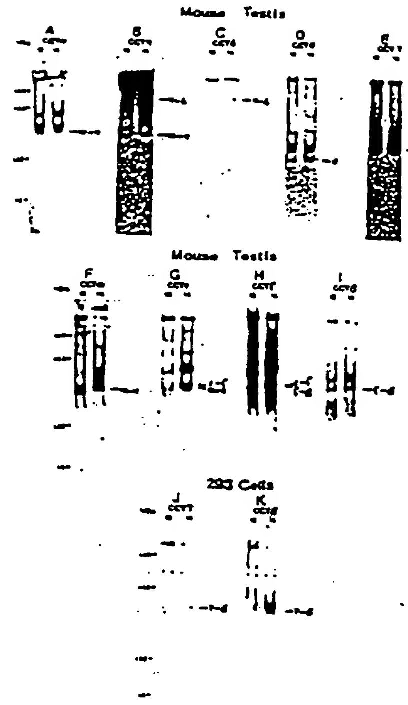

Figure 4 Mouse testis sucrose gradient fractions 13 and 14 (which correspond to sucrose density 1.054 g/cm^3 and 1.039 g/cm^3 respectively) analysed by non-denaturing polyacrylamide gel electrophoresis reveals the subunit-subunit association patterns in CCT micro-complexes.

Sucrose gradient fractions 13 and 14 were resolved in a 6% non-denaturing polyacrylamide gel followed by Western blotting. The CCT subunit-subunit association patterns were determined by probing the blot sequentially with specific antibodies recognising the eight CCT subunits to locate superimposible bands. Panels A-E showed the distribution patterns of CCTα, CCTη, CCTδ, CCTθ and CCTγ respectively, when existing either as components of intact CCT or CCT micro-complexes or present as free subunits, on one Western blot. Panels F-I showed the distribution patterns of CCTα, CCTε, CCTζ and CCTβ respectively when existing either as components of intact CCT or CCT micro-complexes or present as free subunits on another equivalent Western blot. Panels J and K showed the distribution patterns of CCTγ and CCTβ respectively (as components of intact CCT or CCT micro-complexes as well as free subunits) in sucrose gradient fractions 13 and 14 of 293 cells. Inset arrows indicate the corresponding superimposable protein bands and the symbols indicate the two CCT subunit types whose association is inferred by this band. e.g. The band α-η refers to the superimposible band on the blot when probed sequentially with specific antibodies recognising CCTα and CCTη respectively.

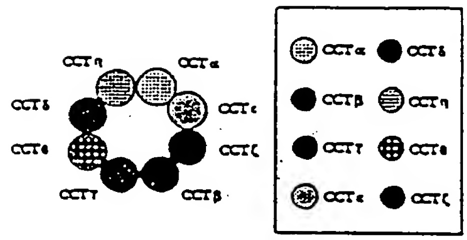

Figure 5 The proposed CCT subunit orientation in each of the two stacked rings in CCT.

Figure 6. Pulse chase analysis of CCT subunits and substrates

Germ cells were prepared from adult male CBA/Ca mice and 5×10^7 cells were labelled with 1mCi of ^{35}S-methionine in 2.5mls HEKRB for 1 hour +/- a 4 hour chase with HEKRB+10mM methionine. Post nuclear supernatants (PNS) were applied to 10.2-40% linear sucrose gradients, centrifuged at 25K rpm for 16 hours 4°C in an SW28 rotor (Beckman) and collected as previously described (Lewis et al., 1992). 3% of the CCT 20S peak fractions were analyzed by 2D-PAGE (Hynes et al., 1995, 1996). Panels A and B show analysis of a peak CCT fraction (19.8% sucrose) from the 1 hour pulse label by silver stain (A) and autoradiogram (B). Note that panel B is the same fraction as panel E in Fig. 1. Panel C shows an autoradiogram of the 20.4% sucrose CCT fraction from the 1 hour pulse label followed by a 4 hour chase. The ^{35}S- counts in each CCT subunit were measured by phosphorimaging. Counts in each CCT subunit were adjusted according to the predicted methionine content from the mouse cDNA sequences (Kubota et al., 1994, 1995b) and are expressed as a proportion of the counts observed in CCTε/S2=1. They are as follows; (1) Panel B: CCTθ/S1=0.24, CCTα/S3=0.9, CCTβ/S4=0.29, CCTγ/S5=0.29. (2) Panel C: CCTθ/S1=0.37, CCTα/S3=1.63, CCTβ/S4=0.64, CCTγ/S5=0.76, CCTζ2/S7=0.58. The efficacy of the pulse-chase conditions is demonstrated by the relative counts in α- and β-tubulin and CCTε in the pulse (Panel B; CCTε=1, α-tubulin=2.85 and β-tubulin=2.01) compared to the chase (Panel C; CCTε=1, α-tubulin=0.45, β-tubulin=0.28.

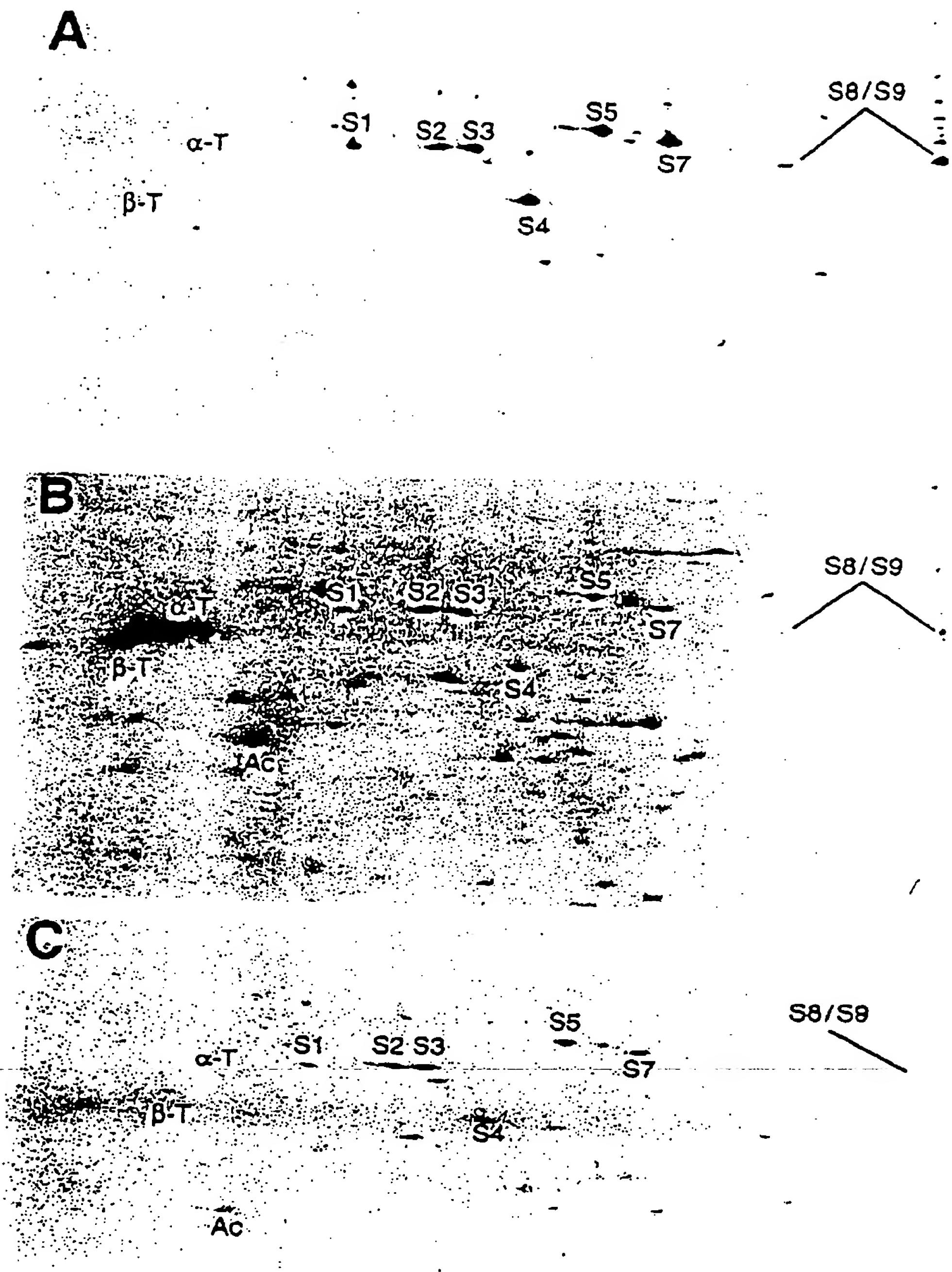
A
S1
S2
S3
S5
S7
S8/S9
α-T
β-T
S4
B
S1
S2
S3
S5
S7
S8/S9
α-T
β-T
S4
Ac
C
S1
S2
S3
S5
S7
S8/S9
α-T
β-T
S4
Ac

Figure 6

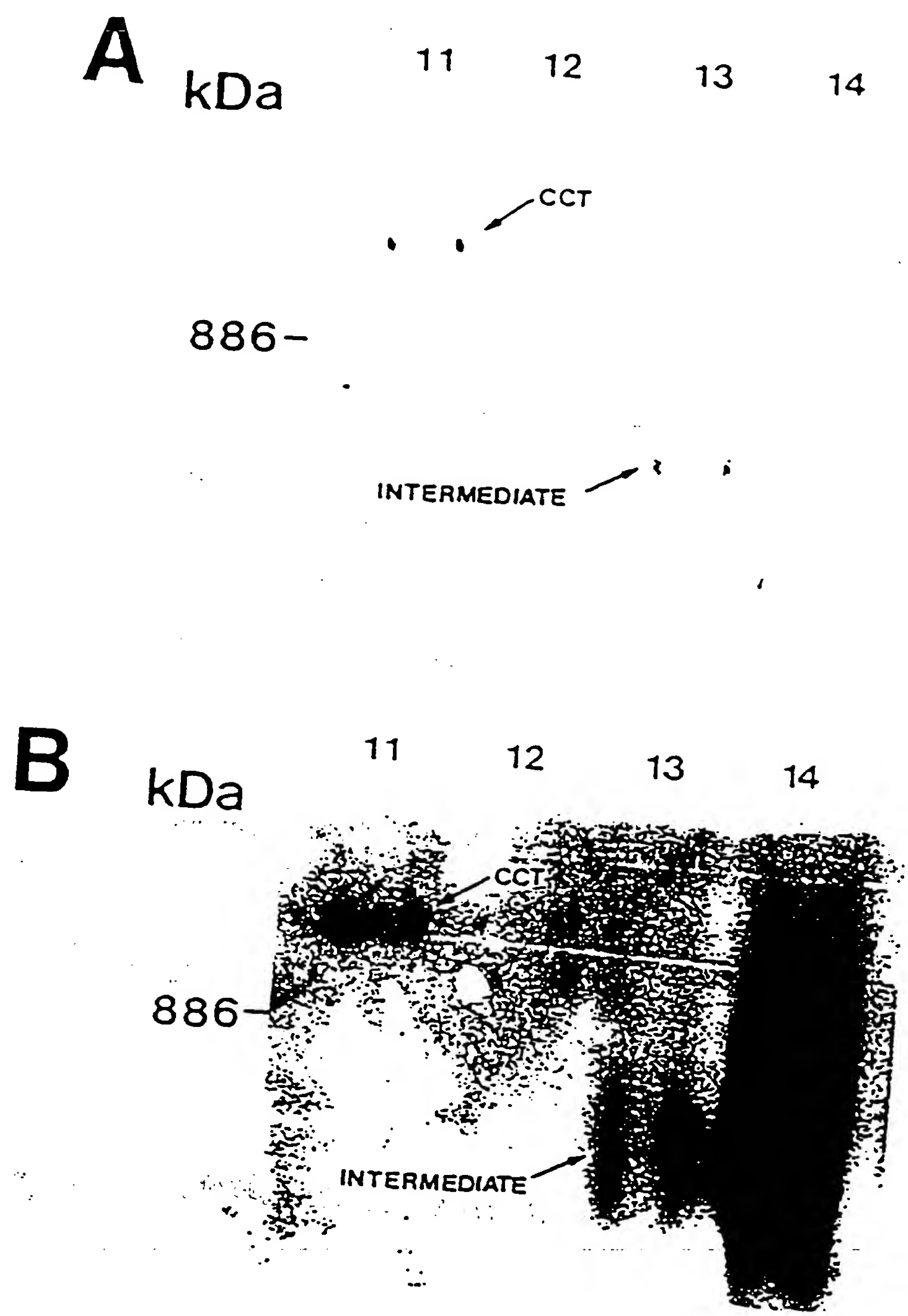

Figure 7 The existence of CCT subunits in the actin intermediate.

(A) Western blot of 6% non-denaturing polyacrylamide gel analysing sucrose fractions 11-14 (correspond to sucrose densities 1.081-1.053 g/cm^3) showing the locations of ^{35}S-labelled β-actin in CCT and the intermediate by autoradiography.

(B) The same western blot showing the locations of CCT subunit species in CCT and the intermediate by probing with polyclonal antibody UM1 which recognises the epitope 'GDGTT' present in all chaperonins (Hynes et al., 1995). Dilution used for th antibody probing with UM1 was 1:2000.

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Figure 8 The ring disassembly of ^{35}S-labelled CCTδ and CCTε containing CCT, into CCTδ and CCTε micro-complexes and/or CCTδ and CCTε monomers visualised in sucrose fractions 3-16 analysed in 6% non-denaturing polyacrylamide gels.

(A) ATP-depleted reticulocyte lysate, reconstituted with CCT, labelled with CCTδ and CCTε, expressing non-labelled human β-actin for 90 minutes. The reaction mix was separated along a continuous 10-40% sucrose gradient and the resultant fractions 3-16 were resolved in a 6% non-denaturing polyacrylamide gel. The distribution of labelled CCT subunits were determined by autoradiography.

(B) and (C) Analysis of sucrose fractions 12 and 14 respectively from (A) by Semi-Native Diagonal Electrophoresis to illustrate the absence of ring disassembly of CCT in the presence of apyrase. Samples from fractions 12 and 14 were resolved by 6% native PAGE followed by 8% SDS PAGE in the perpendicular direction. Only contaminating protein signals were observed by autoradiography.

(D) Fresh reticulocyte lysate, reconstituted with CCT, labelled with CCTδ and CCTε, was used in expressing non-labelled human β-actin and was separated along a continuous 10-40% sucrose gradient with the resultant fractions 3-16 resolved in a 6% non-denaturing polyacrylamide gel. The distribution of labelled CCT subunits was determined by autoradiography

(E) and (F) Analysis of sucrose fractions 12 and 14 respectively from (D) by Semi-Native Diagonal Electrophoresis to illustrate the ring disassembly of CCT because signals representing CCT subunits or micro-complexes were present in both fractions.

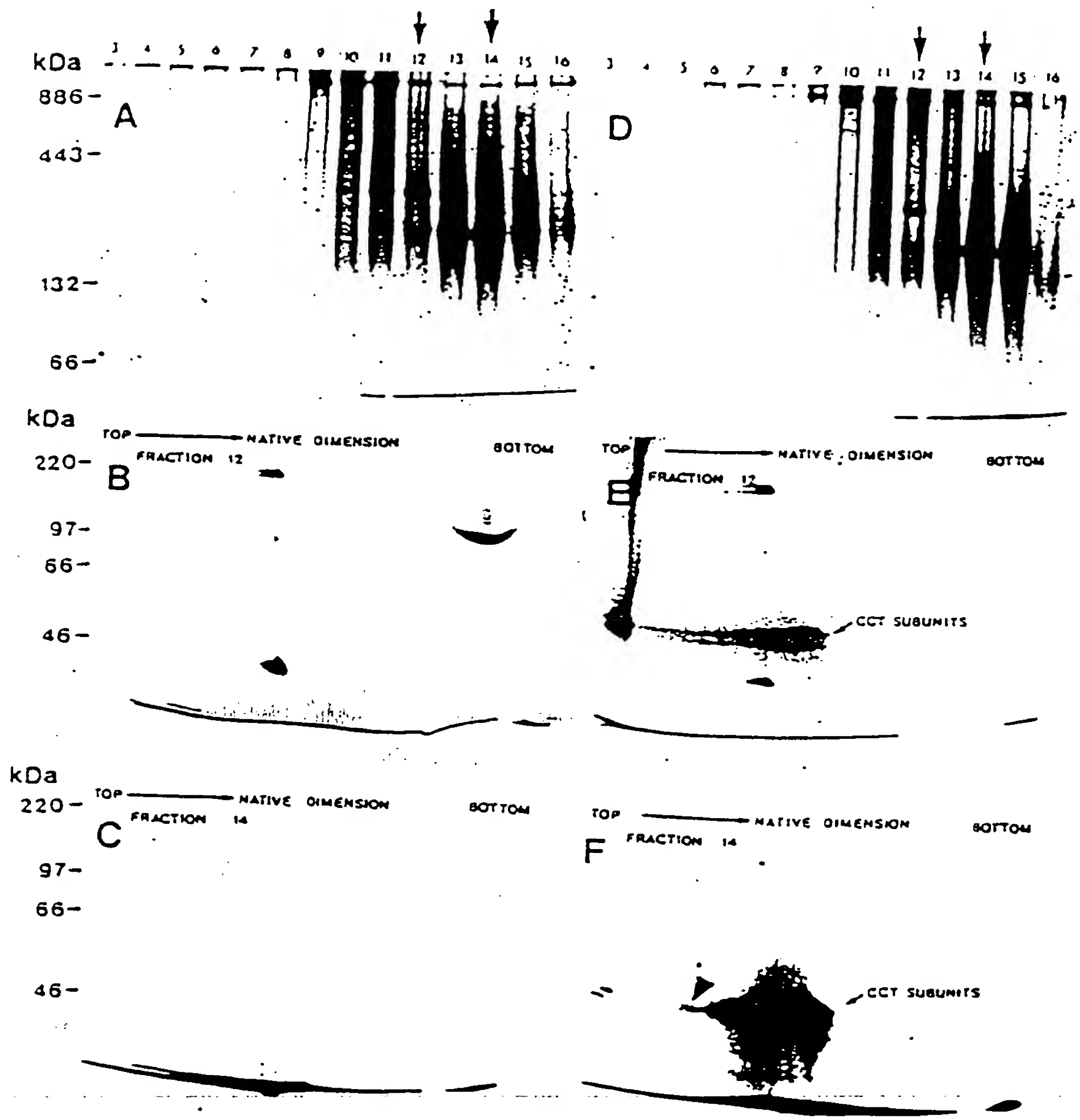

kDa
886
443
132
66
3 4 5 6 7 8 9 10 11 12 13 14 15 16
A
D
kDa
220
97
66
46
TOP
NATIVE DIMENSION
BOTTOM
FRACTION 12
B
E
CCT SUBUNITS
kDa
220
97
66
46
C
F
FRACTION 14
CCT SUBUNITS

Figure 8

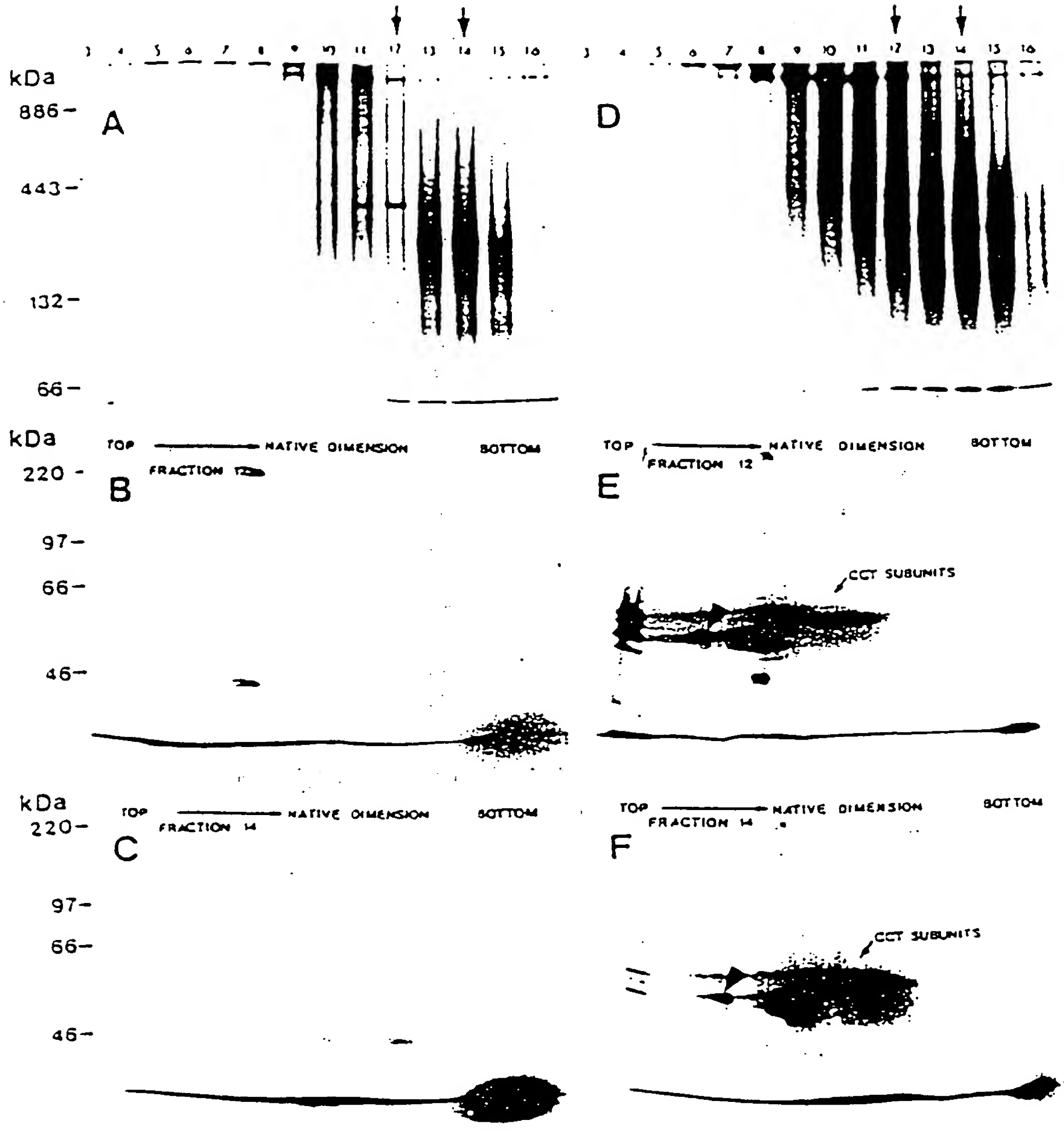
Figure 9 The ring disassembly of ^{35}S-labelled CCTβ, CCTζ, CCTη and CCTθ containing CCT, into CCTβ, CCTζ, CCTη and CCTθ micro-complexes and/or CCTβ, CCTζ, CCTη and CCTθ monomers visualised in sucrose fractions 3-16 analysed in 6% non-denaturing polyacrylamide gels.

(A) ATP-depleted reticulocyte lysate, reconstituted with CCT, labelled with CCTβ, CCTζ, CCTη and CCTθ, expressing non-labelled human β-actin for 90 minutes. The reaction mix was separated along a continuous 10-40% sucrose gradient and the resultant fractions 3-16 were resolved in a 6% non-denaturing polyacrylamide gel. The distribution of labelled CCT subunits were determined by autoradiography.

(B) and (C) Analysis of sucrose fractions 12 and 14 respectively from (A) by Semi-Native Diagonal Electrophoresis to illustrate the absence of ring disassembly of CCT in the presence of apyrase. Samples from fractions 12 and 14 were resolved by 6% native PAGE followed by 8% SDS PAGE in the perpendicular direction. Only contaminating protein signals were observed by autoradiography.

(D) Fresh reticulocyte lysate, reconstituted with CCT, labelled with CCTβ, CCTζ, CCTη and CCTθ, was used in expressing non-labelled human β-actin and was separated along a continuous 10-40% sucrose gradient with the resultant fractions 3-16 resolved in a 6% non-denaturing polyacrylamide gel. The distribution of labelled CCT subunits was determined by autoradiography

(E) and (F) Analysis of sucrose fractions 12 and 14 respectively from (D) by Semi-Native Diagonal Electrophoresis to illustrate the ring disassembly of CCT because signals representing CCT subunits or micro-complexes were present in both fractions.


3 4 5 6 7 8 9 10 11 12 13 14 15 16
kDa
886
443
132
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A
D
kDa
220
97
66
46
TOP
NATIVE DIMENSION
BOTTOM
FRACTION 12
B
E
CCT SUBUNITS
kDa
220
97
66
46
FRACTION 14
C
F

Figure 9

Figure 10 Newly synthesized CCT subunits are incorporated into CCT semi-conservatively.

(A) The protein sequences of the C-termini of rabbit, wildtype and mutant mouse CCTα.

(B) The difference in CCT migration distance induced by monoclonal antibody 23C after incorporating either wildtype mouse CCTα (lane 2) or mutant CCTα (lane 4) is clearly discernable. Lane 1 and 3 represents the migration of CCT without exposure to antibody 23C after incorporating either wildtype mouse CCTα (lane 1) and mutant mouse CCTα (lane 3).

(C) A pictorial representation of the coupling of two antibody molecules onto rabbit endogenous CCT and one antibody molecule coupled onto CCT containing an incorporated mutant mouse CCTα subunit (subunit in black).

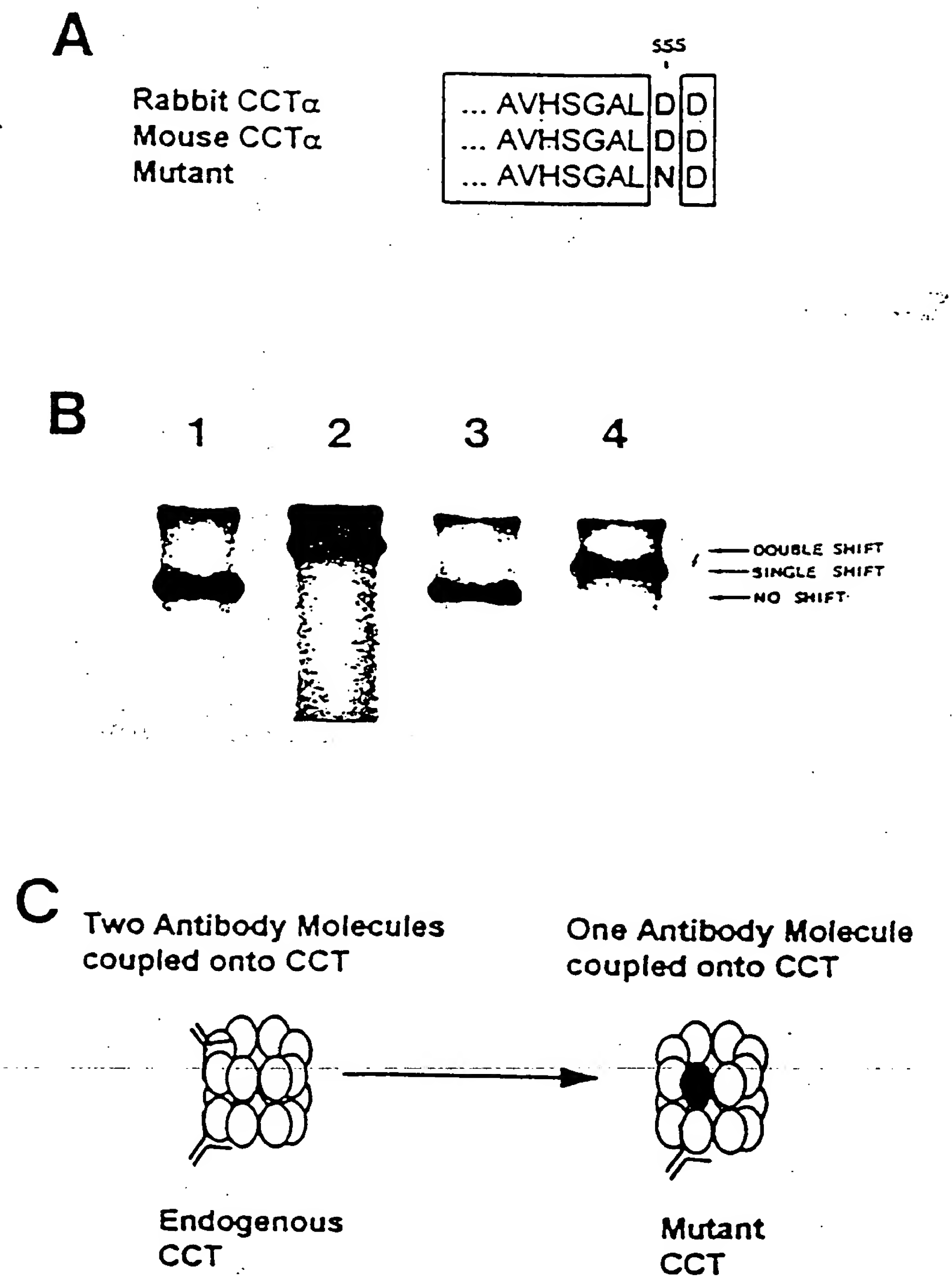
A
sss
Rabbit CCTα
Mouse CCTα
Mutant
... AVHSGAL D D
... AVHSGAL D D
... AVHSGAL N D
B
1
2
3
4
DOUBLE SHIFT
SINGLE SHIFT
NO SHIFT
C
Two Antibody Molecules coupled onto CCT
One Antibody Molecule coupled onto CCT
Endogenous CCT
Mutant CCT

Figure 10

Table 3

mouse beta actin - 15mer peptides with 5 residue overlap :

1,	MDDDIAALVVDNGSG	= 1 - 15
2,	AALVVDNGSGMCKAG	= 6 - 20
3,	DNGSGMCKAGFAGDD	= 11 - 25
4,	MCKAGFAGDDAPRAV	= 16 - 30
5,	FAGDDAPRAVFPSIV	= 21 - 35
6,	APRAVFPSIVGRPRH	= 26 - 40
7,	FPSIVGRPRHQGVMV	= 31 - 45
8,	GRPRHQGVMVGMGQK	= 36 - 50
9,	QGVMVGMGQKDSYVG	= 41 - 55
10,	GMGQKDSYVGDEAQS	= 46 - 60
11,	DSYVGDEAQSKRGIL	= 51 - 65
12,	DEAQSKRGILTLKYP	= 56 - 70
13,	KRGILTLKYPIEHGI	= 61 - 75
14,	TLKYPIEHGIVTNWD	= 66 - 80
15,	IEHGIVTNWDDMEKI	= 71 - 85
16,	VTNWDDMEKIWHHTF	= 76 - 90
17,	DMEKIWHHTFYNELR	= 81 - 95
18,	WHHTFYNELRVAPEE	= 86 - 100
19,	YNELRVAPEEHPVLL	= 91 - 105
20,	VAPEEHPVLLTEAPL	= 96 - 110
21,	HPVLLTEAPLNPKAN	= 101 - 115
22,	TEAPLNPKANREKMT	= 106 - 120
23,	NPKANREKMTQIMFE	= 111 - 125
24,	REKMTQIMFETFNTP	= 116 - 130
25,	QIMFETFNTPAMYVA	= 121 - 135
26,	TFNTPAMYVAIQAVL	= 126 - 140
27,	AMYVAIQAVLSLYAS	= 131 - 145
28,	IQAVLSLYASGRTTG	= 136 - 150
29,	SLYASGRTTGIVMDS	= 141 - 155
30,	GRTTGIVMDSGDGVT	= 146 - 160
31,	IVMDSGDGVTHTVPI	= 151 - 165
32,	GDGVTHTVPIYEGYA	= 156 - 170
33,	HTVPIYEGYALPHAI	= 161 - 175
34,	YEGYALPHAILRLDL	= 166 - 180
35,	LPHAILRLDLAGRDL	= 171 - 185
36,	LRLDLAGRDLTDYLM	= 176 - 190
37,	AGRDLTDYLMKILTE	= 181 - 195
38,	TDYLMKILTERGYSF	= 186 - 200
39,	KILTERGYSFTTTAE	= 191 - 205
40,	RGYSFTTTAEREIVR	= 196 - 210
41,	TTTAEREIVRDIKEK	= 201 - 215
42,	REIVRDIKEKLCYVA	= 206 - 220
43,	DIKEKLCYVALDFEQ	= 211 - 225
44,	LCYVALDFEQEMATA	= 216 - 230

45, LDFEQEMATAASSSS = 221 - 235
46, EMATAASSSSLEKSY = 226 - 240
47, ASSSSLEKSYELPDG = 231 - 245
48, LEKSYELPDGQVITI = 236 - 250
49, ELPDGQVITIGNERF = 241 - 255
50, QVITIGNERFRCPEA = 246 - 260
51, GNERFRCPEALFQPS = 251 - 265
52, RCPEALFQPSFLGME = 256 - 270
53, LFQPSFLGMESCGIH = 261 - 275
54, FLGMESCGIHETTFN = 266 - 280
55, SCGIHETTFNSIMKC = 271 - 285
56, ETTFNSIMKCDVDIR = 276 - 290
57, SIMKCDVDIRKDLYA = 281 - 295
58, DVDIRKDLYANTVLS = 286 - 300
59, KDLYANTVLSGGTTM = 291 - 305
60, NTVLSGGTTMYPGIA = 296 - 310
61, GGTTMYPGIADRMQK = 301 - 315
62, YPGIADRMQKEITAL = 306 - 320
63, DRMQKEITALAPSTM = 311 - 325
64, EITALAPSTMKIKII = 316 - 330
65, APSTMKIKIIAPPER = 321 - 335
66, KIKIIAPPERKYSVW = 326 - 340
67, APPERKYSVWIGGSI = 331 - 345
68, KYSVWIGGSILASLS = 336 - 350
69, IGGSILASLSTFQQM = 341 - 355
70, LASLSTFQQMWISKQ = 346 - 360
71, TFQQMWISKQEYDES = 351 - 365
72, WISKQEYDESGPSIV = 356 - 370
73, EYDESGPSIVHRKCF = 361 - 375
74, GGGGGGPSIVHRKCF = 366 - 375
75, GGGGGGGGGGHRKCF = 371 - 375

Other peptides to include:

76, KYSVWIGGSILASLS
alpha helix in subdomain 1 of rabbit alpha actin- contains two hydrophobic residues accessible to solvent
(residues S338 - S348)

77, PRHQGVMVGMGQKDS
loop in subdomain 2 of rabbit alpha actin- major interaction site with DNase I
(residues P38 - S52)

78, IVLDSGDGVTHNVPI
beta strands in subdomain 3 of rabbit alpha actin
(residues G150 - Y166)

79, LVCDNGSGLVKAGFA

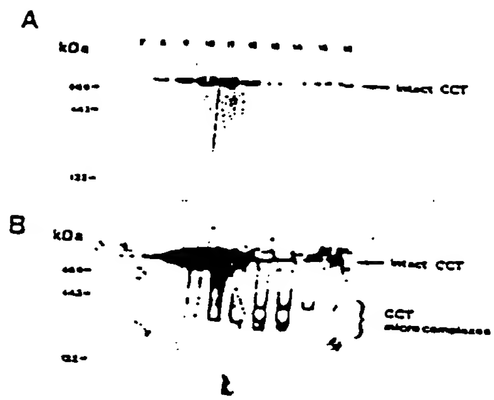

Figure 1 Multiple smaller complexes containing CCTα in sucrose fractions 13-16 (corresponding to sucrose densities 1.054 g/cm^3-1.030g/cm^3).

Mouse testis sucrose gradient fractions 7-16 (corresponding to sucrose densities 1.132 g/cm^3-1.030 g/cm^3) were resolved in a 6% non-denaturing polyacrylamide gel followed by Western blotting. The blot was then probed with monoclonal antibody 91A, which recognises mouse CCTα. The distribution pattern of CCTα is revealed by chemiluminescence and two different exposure times are shown; A) 10 seconds; B) 2 minutes. The presence of smaller complexes containing CCTα is clearly shown in the longer exposure (B).

analagous beta strand motif in subdomain 1 of rabbit alpha actin
(residues L8 - F21)

80, LFQPSFIGMESAGIH
loop in subdomain 4 of rabbit alpha actin- involved in contact across helix axis in F-actin
(residues F262 - I274)

81, TTAEREIVRDIKEKL
alpha helix in subdomain 4 of rabbit alpha actin- minor interaction site with DNase I
(residues T203 - L216)

82, YVGDEAQSKRGILTL
beta alpha beta unit in subdomain 2 of rabbit alpha actin- minor interaction site with DNase I/ hexokinase-like unit
(residues K61 - L65)

83, VMSGGTTMYPGIADR
loop in subdomain 3 of rabbit alpha actin- forms pocket for adenine base of nucleotide
(residues S300 - I309)

84, KIKIIAPPERKYSVW
beta strand and loop in subdomain 3 of rabbit alpha actin- forms pocket for adenine base of nucleotide
(residues K328 - S338)

85, GFAGDDAPRAVFPSI
loop in subdomain 1 of rabbit alpha actin- central contact region of myosin on 'flat' side of actin
(residues F21 - P32)

86, YNELRVAPEEHPTLL
loop in subdomain 1 of rabbit alpha actin- contact region of myosin on 'flat' side of actin
(residues N92 - T103)

87, TFQQMWITKQEYDEA
alpha helices in subdomain 1 of rabbit alpha actin- bind myosin chains
(residues S348 - A365)

88, DEDETTALVCDNGSG
N-terminal 15 residues of rabbit alpha actin- important in binding myosin
(residues D1 - G15)

89, EYDEAGPSIVHRKCF
C-terminal 15 residues of rabbit alpha actin
(residues E361 - F375)

90, SKQEYDESGPSIVHR
truncated C-terminus of mouse beta actin
(residues S358 - R372)

91, ILTERGYSFVTTAER

loop in subdomain 4 of rabbit alpha actin- analagous to DNase I-binding loop in subdomain 2
(residues T194 - T203)

92, ALDFENEMATAASSS
alpha helix flanked by loops in subdomain 4 of rabbit alpha actin
(residues F223 - A230)

93, WDDMEKIWHHTFYNE
alpha helix in subdomain 1 of rabbit alpha actin
(residues W79 - N92)

94, +ve control for 91a = STDLVAKLRAFHNEA

Figure 11

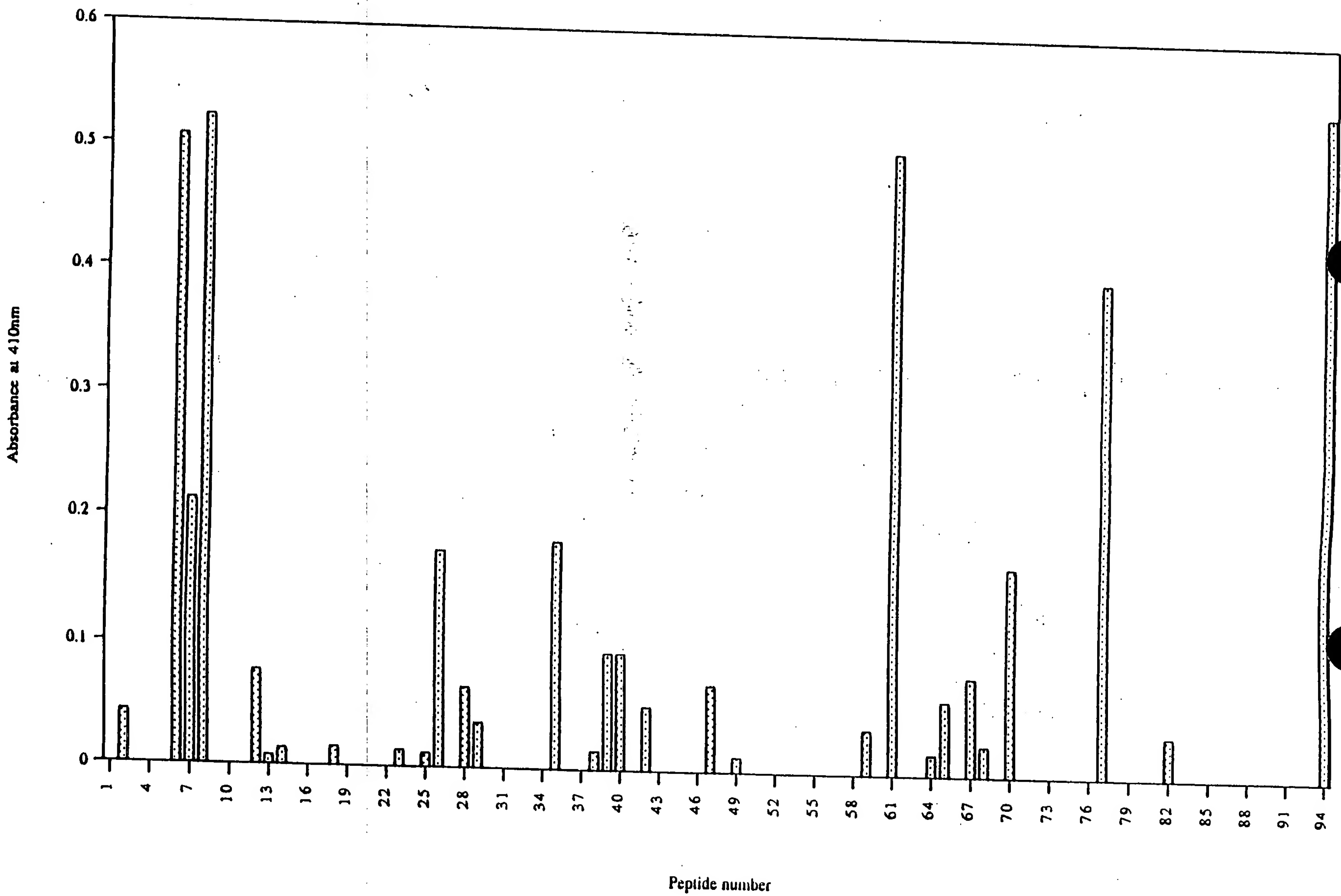
Absorbance at 410nm
0
0.1
0.2
0.3
0.4
0.5
0.6
Peptide number
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76
79
82
85
88
91
94

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Figure 12

Peptide Sequences

Reference Peptide No.	Peptide No. In Figure 11	Peptide Sequence
1	6	APRAVFPSIVGRPRH
2	7	FPSIVGRPRHQGVMV
3	8	GRPRHQGVMVGMGQK
4	61	GGTTMYPGIADRMQK
5	77	PRHQGVMVGMGQKDS
6	26	TFNTPAMYVAIQAVL
7	35	LPHAILRLDLAGRDL
8	70	LASLSTFQQMWISKQ
9	12	DEAQSKRGILTLKYP
10	28	IQAVLSLYASGRTTG
11	39	KILTERGYSFTTTAE
12	40	RGYSFTTTAEREIVR
13	47	ASSSSLEKSYELPDG
14	65	APSTMKIKIIAPPER
15	67	APPERKYSVWIGGSI

Figur 13

Interaction of actin derived peptides and alanin scanning mutations of actin derived p ptides with CCT

Mouse testis CCT was incubated singly or in combination with the following peptides. Peptide sequences are in parenthesis:

Lane 1 Peptide 3 at 1/100	(SGSGAPRAVFPSIVGRPRH)
Lane 2 Peptide 4 at 1/100	(SGSGFPSIVGRPRHQGVMV)
Lane 3 Peptide 5 at 1/100	(SGSGGRPRHQGVMVGMGQK)
Lane 4 Peptide 5 at 1/100	(SGSGGRPRHQGVMVGMGQK)
Lane 5 Peptide 5 at 1/10	(SGSGGRPRHQGVMVGMGQK)
Lane 6 Peptide 5 at 1/100 without CCT	(SGSGGRPRHQGVMVGMGQK)
Lane 7 Peptide 25 at 1/100	(SGSGSTDLVAKLRAFHNEA)
Lane 8 Peptides 3, 4, and 5 at 1/100 each	(SGSGAPRABFPSIVGRPRH)
	(SGSGFPSIVGRPRHQGVMV)
	(SGSGGRPRHQGVMVGMGQK)
Lane 9 Peptide 22 at 1/100	(SGSGAAAAAQGVMVGMGQK)

In all lanes, CCT was incubated with peptide on ice for one hour, with the exception of Lane 5, where CCT was incubated with peptide at room temperature for one hour.

Samples were electrophoresed on 6% native gels, transferred to nitrocellulose membrane and incubated with Neutravidin-HRP (Pierce) at 2 µg per ml to reveal the distribution of biotinylated peptides. The arrowed region shows CCT complexes bound by peptides.

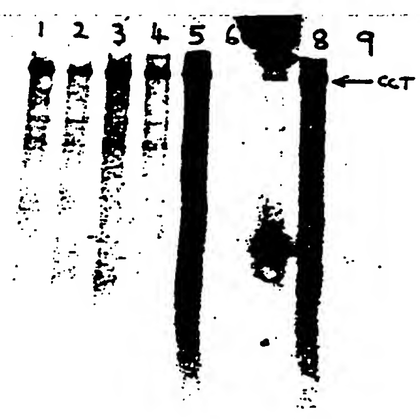

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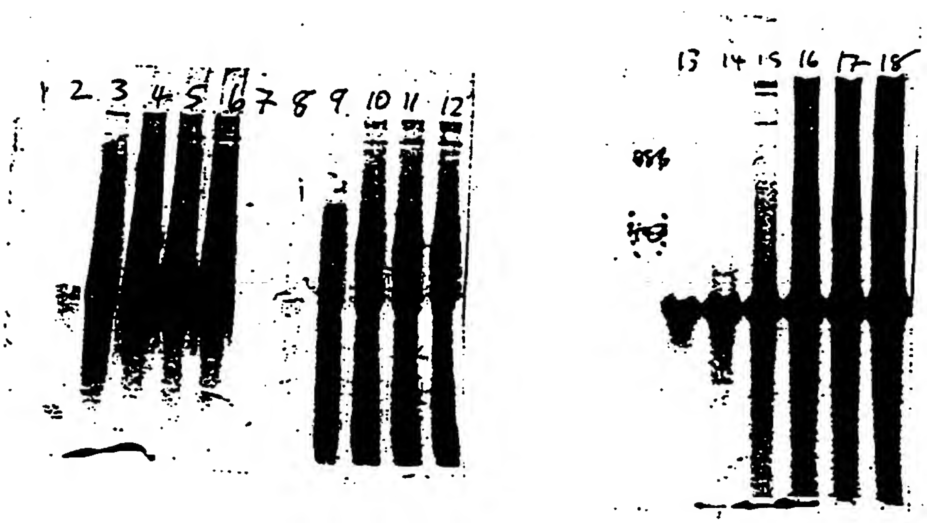
Figure 14

Interaction of cyclin D1 and cyclin E with CCT

p Bluescript plasmids containing full length mouse cyclin D1 cDNA or human cyclin E cDNA were used to programme rabbit reticulocyte lysate transcription translation systems (Liou & Willison, 1997). Time courses of interactions of cyclins with CCT were analyzed on 6% native polyacrylamide gels (Liou & Willison, 1997). At the indicated times, 5 µl aliquots of the lysate reactions were added to 7 µl of 10ml EDTA (ph 8.0) and 4 µl of 4x gel loading buffer and placed on ice.

The lanes 1 - 6 show CCT α at t = 0, 5, 10, 20, 30, 60 minutes. Lanes 7-12 show pBSK CY1 1 (mouse D1) at t = 0, 5, 10, 20, 30, 60 minutes. The right hand panel (lanes 13-18) shows a time course expression of cyclin E at t = 0, 5, 10, 20, 30, 60 minutes. In the right hand panel, the lane marked M shows the migration of molecular weight markers of 886kDa and 443 kDa.

This kinetic analysis shows that cyclins do not appear to be interacting with CCT in a manner resembling bone fide substrates, such as actins and tubulins, but seem to have similar kinetics as the cycling of CCT subunits into rabbit CCT in the lysate. This suggests some regulatory role for the interactions of cyclins with CCT.



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Mewburn Ellis